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CASWELL FILE
009595

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

JUL 13 1992

JUL 13 1992

MEMORANDUM

OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

SUBJECT: Benzyl benzoate: Review of Two Mutagenicity Studies (MRIDs 420231-01 and 420231-02)

REREG CASE #4013
D177617
S417062
Shaw.No. 009501

FROM: Karen L. Hamernik, Ph.D. KLH 6/24/92
Acting Section Head, Review Section III
Toxicology Branch I
Health Effects Division (H7509C)

TO: Ernestine Dobbins
PM Team Reviewer, PM Team #52
Special Review and Reregistration Division (H7508C)

THRU: Karl Baetcke, Ph.D.
Chief, Toxicolgy Branch I
Health Effects Division (H7509C)

Karl D. Baetcke 7/2/92

Conclusions

Both studies had been previously reviewed.

1. MRID 420231-01, **Gene Mutation Assay in Chinese Hamster Ovary (CHO) Cells In Vitro with Benzyl Benzoate,** was judged to be Acceptable and to satisfy Guideline requirements for genetic effects Category I, Gene Mutations, Guideline 84-2(a).

2. MRID 420231-02, **Chromosome Aberration Assay in Human Lymphocytes In Vitro with Benzyl Benzoate,** was judged to be Unacceptable and does not satisfy Guideline requirements for genetic effects Category II, Structural Chromosome Aberrations, Guideline 84-2(b). This study cannot be upgraded.

Action Requested

Reviews of two mutagenicity studies were requested. Phase 3 Summaries for the studies accompanied the data package record. The studies were as follows:

1. MRID 420231-01. **Gene Mutation Assay in Chinese Hamster Ovary (CHO) Cells In Vitro with Benzyl Benzoate.** Study Number: 203422. Testing Facility: CCR-Cytotest Cell Research GmbH and Co., KG, Rossdorf. Sponsor: Werner and Mertz GmbH, Mainz, Germany. Report issued: 12/6/90. (Dose-ranging study MRID 42023001).

2. MRID 420231-02. **Chromosome Aberration Assay in Human Lymphocytes In Vitro with Benzyl Benzoate.** Study Number: 203411. Testing Facility: CCR-Cytotest Cell Research GmbH and Co., KG, Rossdorf. Sponsor: Werner and Mertz GmbH, Mainz, Germany. Report issued: 5/16/91. (Dose-ranging study MRID 42023002).

Toxicology Branch Response

These studies have already been reviewed and the reviews are in Toxicology Branch files. Copies of the reviews are attached to this memo.

MRID 420231-01 was judged to be Acceptable and to satisfy Guideline requirements for genetic effects Category I, Gene Mutations.

MRID 420231-02 was judged to be Unacceptable and does not satisfy Guideline requirements for genetic effects Category II, Structural Chromosome Aberrations. This study cannot be upgraded.

CASWELL FILE

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

009412

OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: Review of Two Mutagenicity Studies on Benzyl Benzoate: Cytogenetic Assay in Human Lymphocytes; Gene Mutation in Cultured Chinese Hamster Lung Cells

Tox Chem No.: 082
HED Project No.: 2-0373
Record No.: S-406128
ID No.: 059820-E
Study Nos.: 203411
203422

TO: Richard Mountfort, PM Team #10
Insecticide Rodenticide Branch
Registration Division (H7505C)

FROM: Brian Dementi, Ph.D., D.A.B.T. Brian Dementi 3/25/92
Review Section III
Toxicology Branch I
Health Effects Division (H7509C)

THRU: Henry Spencer, Ph.D., Acting Section Head 3/26/92
Review Section III
Toxicology Branch I
Health Effects Division (H7509C) KB 3/27/92

ACTION REQUESTED

The Registrant has submitted for review two mutagenicity studies on benzyl benzoate designed to satisfy gene mutation (Study No. 203422) and structural chromosomal aberrations (Study No. 203411) mutagenicity guideline testing requirements.

CONCLUSIONS

(1) Review of the gene mutation study (No. 203422) in cultured Chinese hamster ovary cells (CHO/HGPRT) reveals the study to be acceptable in satisfying the gene mutation guideline requirement.

Under the conditions of the assay, doses of nonactivated benzyl benzoate (10 to 120 ug/ml) and doses of S9-activated

benzyl benzoate (50 to 500 ug/ml) did not induce a mutagenic response in the independent assays. Cytotoxicity was observed in the absence of S9 activation, but cytotoxicity was not observed with S9 activation. It is concluded that benzyl benzoate was tested to the limit of solubility, and to cytotoxic levels without S9 activation, with no evidence of a mutagenic effect.

(2) Review of the cytogenetic assay in human lymphocytes (Study No. 203411) disclosed the study to be unacceptable and, hence, does not serve as intended to satisfy the chromosomal aberrations testing requirement.

Under conditions of the assay, no evidence of a clastogenic effect was seen in the absence of S9 activation. However, no conclusion could be reached when the assay was conducted in the presence of S9 activation. While there were no significant increases in the frequency of cells with aberrations, concerns exist with respect to the presence of complex aberrations at 30 to 500 ug/ml. In the opinion of the reviewers, questionable findings likely would have been resolved had lymphocytes been tested from more than one donor, or possibly if the assay had been repeated. Since the questionable findings remain unresolved, the study is deemed unacceptable.

DATA EVALUATION REPORT

BENZYL BENZOATE

Study Type: Mutagenicity: Gene Mutation in Cultured Chinese Hamster Lung Cells (HGPRT)

Prepared for:

Health Effects Division
Office of Pesticide Programs
Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Clement International Corporation
9300 Lee Highway
Fairfax, VA 22031-1207

Principal Reviewer Lynne T. Haber
Lynne T. Haber, Ph.D. Date 3/3/92

Independent Reviewer Nancy E. McCarroll
Nancy E. McCarroll, B.S. Date 3/3/92

QA/QC Manager Sharon Segal
Sharon Segal, Ph.D. Date 3/9/92

Contract Number: 68D10075
Work Assignment Number: 1-45
Clement Number: 91-148
Project Officer: James Scott

GUIDELINE SERIES 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE GENE MUTATION

MUTAGENICITY STUDIES

EPA Reviewer: Brian Dementi, Ph.D. Signature:
Review Section III,
Toxicology Branch (I)/HED Date:
Acting EPA Section Head: Henry Spencer, Ph.D. Signature:
Review Section III,
Toxicology Branch (I)/HED Date: 5/23/92

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Gene mutation in cultured Chinese hamster ovary cells (CHO/HGPRT)

EPA IDENTIFICATION Numbers:

Tox Chem. Number: 082

MRID Number: 420231-01

TEST MATERIAL: Benzyl benzoate

SYNONYMS: Benzoic acid benzyl ester; active ingredient of Acarosan; CAS No. 120-51-4

SPONSOR: Werner and Mertz GmbH, Mainz, Germany

STUDY NUMBER: 203422

TESTING FACILITY: CCR-Cytotest Cell Research GmbH and Co., KG, Rossdorf, Germany

TITLE OF REPORT: Gene Mutation Assay in Chinese Hamster Ovary (CHO) Cells In Vitro With Benzyl Benzoate[1]

AUTHOR: Heidemann, A.

REPORT ISSUED: December 6, 1990

CONCLUSIONS-EXECUTIVE SUMMARY: Under the conditions of the Chinese hamster lung cell HGPRT forward gene mutation assay, doses of nonactivated benzyl benzoate (10 to 120 µg/mL), and doses of S9-activated benzyl benzoate (50 to 500 µg/mL) did not induce a mutagenic response in two independent assays. The test material precipitated at levels above 50 µg/mL +/- S9. Marked cytotoxicity was observed at all nonactivated doses ≥60 µg/mL in the first trial,

[1]There appears to be a discrepancy between the title of the report and the description within the report regarding the actual cell line that was used. While the title indicates Chinese hamster ovary (CHO) cells, the report largely refers to V79 cells. Based on the medium used and the treatment protocol, we assume that Chinese hamster lung V79 cells were assayed.

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and at 150 µg/mL in the second trial. The S9-activated test material was not cytotoxic. Based on these findings, it was concluded that benzyl benzoate was tested to the limit of solubility, and to cytotoxic levels without S9 activation, with no evidence of a mutagenic effect. The study, therefore, satisfies Guideline requirements for genetic effects Category I, Gene Mutations.

STUDY CLASSIFICATION: The study is acceptable.

A. MATERIALS:

1. Test Material: Benzyl benzoate

Description: Colorless liquid
Identification No.: Batch no. 18504
Purity: 99%
Receipt date: Not reported
Stability: 12 months, pure and in solution; expiration date, June 28, 1991
Contaminants: None listed
Solvent used: Ethanol
Other provided information: Stored at room temperature. The frequency of dosing solution preparation was not reported. The stability of the test material in medium was determined.

2. Control Materials:

Negative: Dulbecco's minimal essential medium (DMEM)/F12 supplemented with 10% fetal calf serum (FCS)

Solvent/volume: Ethanol/1% v/v

Positive: Nonactivation (concentration, solvent): Ethyl methanesulfonate (EMS) was prepared in culture medium to yield a final concentration of 1 mg/mL.

Activation (concentration, solvent): 7,12-dimethylbenz(a)anthracene (DMBA) was prepared in dimethyl sulfoxide (DMSO) and used at 15.4 µg/mL

3. Activation: S9 derived from 8-12 week-old male Wistar

x Aroclor 1254	x induced	x rat	x liver
____ phenobarbital	____ noninduced	____ mouse	____ lung
____ none		____ hamster	____ other
____ other		____ other	

The S9 (Lot no. 191289) was prepared by the testing laboratory. The protein content was determined and found to be 30.3 mg/mL.

S9 mix composition:

Component	Concentration in S9 Mix
NADP	4 mM
Glucose 6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Sodium phosphate buffer, pH 7.4	100 mM
S9 homogenate (final protein concentration in cultures)	0.3 mg/mL

4. Test Cells: Mammalian cells in culture

_____ mouse lymphoma L5178Y cells
_____ Chinese hamster ovary (CHO) cells
__x__ V79 cells (Chinese hamster lung fibroblasts)
_____ other (list):

Properly maintained? Yes.
Periodically checked for mycoplasma contamination? Yes.
Periodically checked for karyotype stability? Yes.
Periodically "cleansed" against high spontaneous background? Not reported.

5. Locus Examined:

_____ thymidine kinase (TK)
Selection agent: (give concentration)
_____ bromodeoxyuridine (BrdU)
_____ fluorodeoxyuridine (FdU)

__x__ hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)
Selection agent: (give concentration)
_____ 8-azaguanine (8-AG)
__11 µM__ 6-thioguanine (6-TN)

_____ Na^+/K^+ATPase
Selection agent: (give concentration)
_____ ouabain

_____ other (locus and/or selection agent; give details):

6. Test Compound Concentrations Used:

(a) Preliminary cytotoxicity assay: Eight doses (0.1, 1.0, 10, 30, 60, 100, 250, and 500 µg/mL) were evaluated with and without S9 activation.

(b) Mutation assay:

Without S9 activation: Eight doses (10, 50, 60, 75, 80, 90, 100, and 120 µg/mL) were evaluated in the initial assay, and five doses (50, 90, 100, 120, and 150 µg/mL) were evaluated in the confirmatory assay.

With S9 activation: Four doses (50, 100, 250, and 500 µg/mL) were evaluated in both the initial and confirmatory assays.

B. TEST PERFORMANCE:

1. Cell Treatments:

(a) Cells exposed to test compound for:
__4__ hours (nonactivated) __4__ hours (activated)

(b) Cells exposed to positive controls for:
__4__ hours (nonactivated) __4__ hours (activated)

(c) Cells exposed to negative and/or solvent controls for:
__4__ hours (nonactivated) __4__ hours (activated)

(d) After washing, cells cultured for __7__ days (expression period) before cell selection.

(e) After expression, cells cultured for __8__ days in selection medium to determine numbers of mutants and for __7__ days without selection medium to determine cloning efficiency.

2. Protocol: Not provided.

C. REPORTED RESULTS:

1. Stability Determination: RCC Umweltchemie GmbH and Co., D6101 Rossdorf, Germany, determined the stability of doses ranging from 9.9 to 49.7 µg/mL in DMEM (-FCS) at room temperature and at 37°C. No loss of any of the doses of benzyl benzoate was observed up to 4 hours of incubation.

1. Preliminary Cytotoxicity Assay: Eight doses of the test material (0.1 to 500 µg/mL) were evaluated with and without S9 activation. The solubility limit was 500 µg/mL. Nonactivated benzyl benzoate reduced the relative initial survival (RIS) at 250 µg/mL to 65.4%, and to 5.9% at 500 µg/mL. No cytotoxicity was observed at lower nonactivated doses, or at any S9-activated dose up to the solubility limit.

2. Mutation Assay: Doses for the mutation assays were chosen so that the high dose would reduce the plating efficiency to 20-50%. The study author stated that the first two assays without S9 activation were repeated, since cytotoxicity at levels >100 µg/mL prevented the evaluation of a sufficient number of doses; data from these experiments were not provided. Accordingly, benzyl benzoate was evaluated in the first successful nonactivated mutation assay at six doses ranging from 10 to 120 µg/mL; four S9-activated doses ranging from 50 to 500 µg/mL were also evaluated. The author reported that precipitation of the test material was observed at concentrations above 50 µg/mL. No explanation was provided for the differences in solubility

between the preliminary cytotoxicity assay and the mutation assay. Benzyl benzoate was not cytotoxic at doses ≤50 µg/mL -S9. Cytotoxicity at higher nonactivated concentrations (60-120 µg/mL) was not dose-dependent; RIS was ≤18.6% at all levels. The author attributed the lack of a dose-dependent effect to the insolubility of the test compound. In the presence of S9 activation, benzyl benzoate was not cytotoxic at any tested dose. There was no evidence of a mutagenic effect of benzyl benzoate at any assayed concentration with or without S9 activation (Table 1). In contrast, the positive controls (EMS at 1 mg/mL and DMBA at 15.4 µg/mL) induced marked increases in the number and frequency of mutants.

In the confirmatory assay, the test material was investigated at 50, 90, 100, 120, and 150 µg/mL -S9 and at 50, 100, 250, and 500 µg/mL +S9. RIS for cultures exposed to nonactivated benzyl benzoate ranged from 64.4% at the low dose (50 µg/mL) to 21.8% at the high dose (150 µg/mL); the cytotoxic response was dose-related (Table 2). Severe cytotoxicity (i.e. <50 cells recovered 7 days postseeding) was reported for the cultures treated with 150 µg/mL -S9 after the expression period. As in the first assay, no cytotoxicity was observed at any S9-activated dose. Also in agreement with the findings of the initial assay, the test material was not mutagenic at any nonactivated or S9-activated dose. Our reviewers noted that for both trials, the absolute survival of the solvent control cultures was borderline acceptable (≤59.2% +/- S9). Nevertheless, from the overall results, the study author concluded that benzyl benzoate was not mutagenic in this test system.

D. REVIEWERS' DISCUSSION AND INTERPRETATION OF RESULTS: We assess that the study author's interpretation of the data was correct. Benzyl benzoate was tested to the limit of solubility, and to cytotoxic doses without S9 activation, but showed no evidence of inducing forward mutations at the HGPRT locus in V79 cells. The response of the test system to the positive controls indicated that the assay was sufficiently sensitive to detect a mutagenic response. We, therefore, conclude that benzyl benzoate was not mutagenic in this assay.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLP? Yes. (A quality assurance statement was signed and dated April 24, 1991.)

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 12-19.

TABLE 1. Representative Results of the Initial V79 Chinese Hamster Lung Cell Forward Gene Mutation Assay with Benzyl Benzoate

Substance	Dose/mL	S9-Activation	Relative % Survival (after treatment)[a]	Number of Survivors x10^5 at Selection[a]	Mean Number of Mutant Colonies ±S.D.[b]	Mutation Frequency/ 10^6 cells[c]
Negative Control						
Culture medium	--	-	100 (57.4)[d]	2.38	0.6±0.5	2.5
	--	+	100 (56.6)	3.63	1.2±0.8	3.3
Solvent Control						
Test Material						
Ethanol	1%	-	100 (56.6)	2.24	1.4±0.9	6.2
	1%	+	100 (52.9)	4.07	3.4±1.3	8.4
Positive Control +S9						
Dimethyl sulfoxide	1%	+	100 (52.3)	5.87	2.0±1.0	3.4
Positive Controls						
Ethylmethane sulfonate	1 mg	-	53.1	1.21	57.8±7.9	476.9
7,12-Dimethylbenz-(a)anthracene	15.4 µg	+	28.3	3.37	19.4±5.0	57.6
Test Material						
Benzyl benzoate	50 µg[e]	-	101.8	2.36	2.0±1.9	8.5
	60 µg	-	8.3	2.35	2.4±0.9	10.2
	120 µg[f]	-	18.6	2.01	1.2±0.8	6.0
	50 µg[e]	+	102.1	4.41	2.0±1.4	4.5
	100 µg	+	98.7	3.82	3.8±1.1	9.9
	250 µg	+	100.9	1.78	0.8±0.8	4.5
	500 µg	+	99.4	4.17	0.6±0.9	1.4

[a]Average of two dishes.

[b]Means and standard deviations of five dishes per dosing group.

[c]Mutation Frequency (MF) = $\frac{\text{Mean Number of Mutant Colonies}}{\text{Average Number of Survivors at Selection}}$

[d]Values in parentheses are the absolute survival rates.

[e]Levels >50 µg/mL precipitated. The lowest dose -S9 (10 µg/mL) showed no evidence of a mutagenic effect.

[f]Intermediate doses (75, 80, and 90 µg/mL) exhibited similar cytotoxicity and did not suggest a mutagenic effect.

TABLE 2. Representative Results of the Confirmatory V79 Chinese Hamster Lung Cell Forward Gene Mutation Assay with Benzyl Benzoate

Substance	Dose/mL	S9-Activation	Relative % Survival (after treatment)[a]	Number of Survivors x10^5 at Selection[a]	Mean Number of Mutant Colonies ±S.D.[b]	Mutation Frequency/ 10^6 cells[c]
Negative Control						
Culture medium	--	-	100 (55.7)[d]	3.07	1.6±0.5	5.2
	--	+	100 (51.9)	2.34	1.8±1.3	7.7
Solvent Control						
Test Material						
Ethanol	1%	-	100 (59.2)	2.89	3.4±2.3	11.8
	1%	+	100 (56.3)	2.62	2.4±1.1	9.2
Positive Control +S9						
Dimethyl sulfoxide	1%	+	100 (55.3)	2.84	1.2±0.8	4.2
Positive Controls						
Ethylmethane sulfonate	1 mg	-	65.0	0.40	20.2±7.2	510.1
7,12-Dimethylbenz-(a)anthracene	15.4 µg	+	75.8	1.11	18.4±2.4	165.9
Test Material						
Benzyl benzoate	120 µg[e,f]	-	41.2	2.44	1.6±0.9	6.6
	50 µg[e]	+	89.7	3.12	1.8±1.3	5.8
	250 µg	+	97.3	3.49	2.0±1.6	5.7
	500 µg	+	94.1	2.80	0.2±0.4	0.7

[a]Average of two dishes.

[b]Means and standard deviations of five dishes per dosing group.

[c]Mutation Frequency (MF) = $\frac{\text{Mean Number of Mutant Colonies}}{\text{Average Number of Survivors at Selection}}$

[d]Values in parentheses are the absolute survival rates.

[e]Levels >50 µg/mL precipitated. Intermediate doses (90 and 100 µg/mL -S9, and 100 µg/mL +S9) showed no evidence of a mutagenic effect.

[f]The 50 µg/mL cultures were discarded at day 6 for an unexplained reason. The highest dose (150 µg/mL) was severely cytotoxic after expression.

APPENDIX A

MATERIALS AND METHODS
CBI pp. 12-19

benzyl benzoate

Page_____ is not included in this copy.

Pages 14 through 21 are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

____ Identity of the source of product ingredients.

____ Sales or other commercial/financial information.

____ A draft product label.

____ The product confidential statement of formula.

____ Information about a pending registration action.

X FIFRA registration data.

____ The document is a duplicate of page(s) _____.

____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

BENZYL BENZOATE

Study Type: Mutagenicity: Mammalian Cells in Culture Cytogenetic Assay in Human Lymphocytes

Prepared for:

Health Effects Division
Office of Pesticide Programs
Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Clement International Corporation
9300 Lee Highway
Fairfax, VA 22031-1207

Principal Reviewer Nancy E. McCarroll, B.S. Date 2-28-92

Independent Reviewer Lynne T. Haber, Ph.D. Date 2/28/92

QA/QC Manager Sharon Segal, Ph.D. Date 2/28/92

Contract Number: 68D10075
Work Assignment Number: 1-45
Clement Number: 91-149
Project Officer: James Scott

GUIDELINE SERIES 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE CYTOGENETICS

EPA Reviewer: Brian Dementi, Ph.D. Signature: Brian Dementi
EPA Review Section III Date: 3/5/92
Toxicology Branch (I)/HED
EPA Acting Section Head: Henry Spencer, Ph.D. Signature: H Spencer
EPA Review Section III, Date: 3/23/92
Toxicology Branch (I)/HED

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Mammalian cells in culture cytogenetic assay in human lymphocytes

EPA IDENTIFICATION Numbers:

Tox Chem. Number: 082

MRID Number: 420231-02

TEST MATERIAL: Benzyl benzoate

SYNONYMS: Acarosan®; benzoic acid benzyl ester

SPONSOR: Werner and Mertz GmbH, Mainz, Germany

STUDY NUMBER: 203411

TESTING FACILITY: CCR-Cytotest Cell Research GmbH and Co., KG, Rossdorf, Germany

TITLE OF REPORT: Chromosome Aberration Assay in Human Lymphocytes In Vitro with Benzyl Benzoate

AUTHOR: A. Heidemann

REPORT ISSUED: May 16, 1991.

CONCLUSIONS-EXECUTIVE SUMMARY: Human lymphocytes derived from a single donor were evaluated for chromosome aberrations 24 hours postexposure to three non-activated doses (10.0, 100.0, and 250.0 µg/mL) and three S9-activated doses (30.0, 250.0, and 500.0 µg/mL) of benzyl benzoate. Chromosome aberrations were also scored in cultures exposed to the high dose with and without S9 activation 48 hours posttreatment. Results indicated that levels ≥100.0 µg/mL +/-S9 were partially insoluble in culture medium and that nonactivated 500 µg/mL was cytotoxic. No evidence of a clastogenic effect was seen in the absence of S9 activation. However, no conclusions can be reached for the S9-activated phase of testing. Although there were no significant increases in the freqency of cells with aberrations, our reviewers have concerns

regarding the presence of complex aberrations at 30, 250, and 500 µg/mL (24-hour harvest). We assess that the biological significance, if any, of these findings probably would have been resolved had the study author followed the recommended approach of using human lymphocytes derived from independent donors or by repeating the assay. Since no definitive conclusions can be reached, the study does not satisfy Guideline requirements for genetic effects, Category II, Structural Chromosome Aberrations.

STUDY CLASSIFICATION: The study is unacceptable.

A. MATERIALS:

1. Test Material: Benzyl benzoate

Description: Colorless liquid
Identification No.: Batch number 18504
Purity: 99%
Receipt date: Not reported
Stability: Stable for 12 months in solution; expiration date June 28, 1991
Contaminants: None listed
Solvent used: Ethanol (ETOH)
Other provide information: The test material was stored at room temperature protected from light. Solutions of the test material were prepared on the day of use. The test material was found to be stable in culture medium Dulbecco's modified Eagle medium/Ham's F_{12}, 1:1 (DMEN/F_{12}) at 37°C for 4 hours.

2. Control Materials:

Negative: DMEM F_{12}

Solvent/final concentration: ETOH/1%

Positive: Nonactivation (concentrations, solvent): Ethyl methanesulfonate (EMS) was prepared in DMEM/F_{12} to yield a final concentration of 720 µg/mL.

Activation (concentrations, solvent): Cyclophosphamide (CP) was prepared in DMEM/F_{12} to yield a final concentration of 60 µg/mL.

3. Activation: S9 derived from Wistar, strain WU, male (8-12 weeks of age)

x Aroclor 1254	x induced	x rat	x liver
____ phenobarbital	____ noninduced	____ mouse	____ lung
____ none		____ hamster	____ other
____ other		____ other	

The rat S9 liver homogenate was prepared by the performing laboratory. The protein content of the batch (lot number 191289) used in this study was 30.3 mg/mL.

S9 mix composition:

Component	Concentration in S9 Mix
Sodium phosphate buffer, pH 7.4	100 mM
KCl	33 mM
NADP	4 mM
Glucose 6-Phosphate	5 mM
$MgCl_2$	8 mM
S9	150 mg protein

Note: 20 µl of the S9 mix were added to 10 mL of culture medium to yield a final protein concentration of 0.3 mg/mL.

4. Test Compound Concentration Used:

(a) Preliminary cytotoxicity assay: Cytotoxicity was assessed in parallel with the cytogenetic assay.

(1) Nonactivated conditions: Eight doses (0.3, 1.0, 3.0, 10.0, 30.0, 100.0, 250, and 500.0 µg/mL) with a 24-hour cell harvest and six dose (3.0, 10.0, 30.0, 100.0, 250.0, and 500.0 µg/mL) with a 48-hour cell harvest.

(2) S9-activated conditions: As above.

(b) Cytogenetic assay:

(1) Nonactivated conditions: Cultures in the cytotoxicity phase of testing that were exposed to 10, 100, and 250 µg/mL (24-hour harvest) and to 250 µg/mL (48-hour harvest) were scored for chromosome aberrations.

(2) S9-activated conditions: Cultures in the cytotoxicity phase of testing that were exposed to 30, 250, and 500 µg/mL (24-hour harvest) and to 500 µg/mL (48-hour harvest) were scored for chromosome aberrations.

5. Test Cells: Human lymphocytes were obtained from the blood of one healthy female donor (age 41 years). Lymphocyte cultures were initiated within 24 hours of collection in DMEM/F_{12} medium supplemented with 15% fetal calf serum (FCS) and containing phytohemagglutinin (concentration not specified) and antibiotics.

Properly maintained? Yes.

Cell line or strain periodically checked for mycoplasma contamination? Not applicable.

Cell line or strain periodically check for karyotype stability? Not applicable.

B. TEST PERFORMANCE:

1. Cell Treatments:

(a) Cells exposed to test compound for:
4 hours (nonactivated) 4 hours (activated)

(b) Cells exposed to positive controls for:
4 hours (nonactivated) 4 hours (activated)

(c) Cells exposed to negative and/or solvent controls for:
4 hours (nonactivated) 4 hours (activated)

2. Cytogenetic Assay:

(a) Treatment: Forty-eight hours after initiation, duplicate cultures were exposed to the selected test material doses, the solvent control (ETOH), or the positive controls (EMS or CP) in both the presence and absence of S9 activation. At the end of the 4-hour treatment, cells were centrifuged, refed culture medium, and reincubated. Colcemid (final concentration, 0.2 µg/mL) was added 3 hours before the cultures were harvested (24 and 48 hours posttreatment). Metaphase cells were collected, swollen in 0.0375 M KCl, and fixed in glacial acetic acid: absolute methanol (1:3). Slides were stained with Giemsa and coded.

(b) Metaphase analysis: Two hundred metaphase plates (100 cells/culture) from each selected dose group and the negative, solvent, and positive control groups were scored for chromosome aberrations; gaps were recorded but not included in the aberration frequencies. The mitotic index (MI) was determined by counting 1000 cells per culture. Polyploid cells per 100 scored cells were also determined.

(c) Statistical methods: The data from the experimental groups were evaluated for statistical significance ($p<0.05$) by the Chi-square test.

(d) Evaluation criteria:

(1) Assay validity: The assay was considered acceptable if (a) the frequency of chromosome aberrations in the negative and/or solvent control cultures was within the performing laboratory's historical range (not provided) and (b) the positive controls induced significant increases in the frequency of aberrations.

(2) Positive response: The test material was considered positive if at least one dose caused a significant increase in the chromosome aberrations frequency compared to the negative control.

3. Protocol: None provided.

C. REPORTED RESULTS:

1. Cytotoxicity Assay. Initially doses ranging from 0.3 to 500.0 µg/mL +/-S9 were assessed for cytotoxic effects 24 and 48 hours posttreatment. Slight compound precipitation was reported at concentrations ≥100.0 µg/mL +/-S9.

In the absence of S9 activation, the MI for cells sampled 24 hours postexposure to 500 µg/mL was markedly reduced (~75%) compared to the solvent value; a slight reduction was also seen at 250 µg/mL. Below 250 µg/mL, the MI was not adversely affected by compound treatment. There was an ~40% reduction in mitotic cell recovery 48 hours following treatment with 500 µg/mL -S9; no convincing evidence of cytotoxicity was seen at lower nonactivated doses. No adverse effects on the number of mitotic cells were seen with the S9-activated test material at either harvest time. Based on these preliminary findings, cultures exposed to nonactivated 10.0, 100.0, and 250.0 µg/mL (24-hour cell harvest) and 250.0 µg/mL (48-hour cell harvest) were examined for chromosome aberrations. As the results presented in Table 1 indicated, no significant increases in the frequency of structural chromosome aberrations were found. Similarly, the incidence of numerical aberrations in treated groups were generally comparable to the negative and solvent control values.

In the presence of S9 activation, the test material was not cytotoxic at any level; accordingly, cultures exposed to 30, 250, 500 µg/mL (24-hour sampling time) and 500 µg/mL (48-hour sampling time) were examined for abnormal chromosome morphology (Table 2). In agreement with the nonactivated findings, there were no significant increases in either structural or numerical chromosome aberrations at any S9-activated dose. Our reviewers noted, however, that single complex aberrations were scored at two dose levels following the 24-hour harvest (1 dicentric at 30 µg/mL and 1 exchange figure at 500 µg/mL). Similarly, multiple aberrations (cells with >5 aberrations) were observed at two doses (250 and 500 µg/mL) 24-hour posttreatment.

By convention, multiple aberrations are generally considered to be cells with ≥10 aberrations and the types of aberrations within these cells are rarely identified. However, information accompanying the individual culture data indicated that all abnormal figures in cells classified as multiple aberrations were exchanges. Therefore, the actual number of complex aberrations scored in the 250- and 500-µg/mL treatment groups were >5 and >6 exchanges, respectively.

TABLE 1. Representative Results from the Nonactivated Human Lymphocyte In Vitro Cytogenetic Assays with Benzyl Benzoate

Substance	Dose/mL	Harvest Time (Hours)	Mitotic Index (%)[a]	No. of Cells Scored	Total No. of Aberrations[b]	No of Cells with Aberrations[b]	Percent Cells with Aberrations[b]	Biologically Significant Aberrations (No./Type)[c]
Negative Control								
Culture medium	--	24	4.1	200	3	3	1.50	1B; 1F; 1IF
Solvent Control								
Ethanol	1%	24	4.7	200	2	2	1.00	1B; 1IF
	1%	48	13.6	200	1	1	0.50	1B
Positive Control								
Ethyl methanesulfonate	720 µg	24	5.9	200	27	21	10.50	3B; 8F; 1IF; 15E
Test Material								
Benzyl benzoate	100.0 µg	24[d]	5.1	200	>5	1	0.5	1MA
	250.0 µg	24	3.7	185[e]	7	4	2.16	2B; 5IF
	500.0 µg	24	1.2[g]	--	--	--	--	--
	250.0 µg	48	11.5	100[g]	1	1	1.00	1F
	500.0 µg	48	8.2	ND[h]	--	--	--	--

[a]Number of metaphases per 1000 cells scored per culture.

[b]Gaps excluded.

[c]Abbreviations used:

B = Break
F = Fragments
IF = Isofragment
E = Exchange
MA = Multiple aberration (cells with >5 aberrations; all observed aberrations were exchanges)

[d]No structural aberrations were seen in the lowest scored dose (10 µg/mL). benzo(a)pyrene

[e]Less than 200 metaphase found for analysis.

[f]Insufficient number of metaphases available for analysis.

[g]One culture lost owing to an unspecified technical error.

[h]ND = Not done

TABLE 2. Representative Results from the S9-Activated Human Lymphocyte In Vitro Cytogenetic Assays with Benzyl Benzoate

Substance	Dose/mL	Harvest Time (Hours)	Mitotic Index (%)[a]	No. of Cells Scored	Total No. of Aberrations[b]	No of Cells with Aberrations[b]	Percent Cells with Aberrations[b]	Biologically Significant Aberrations (No./Type)[c]
Negative Control								
Culture medium	--	24	7.7	200	4	4	2.00	2B; 1F; 1IF
Solvent Control								
Ethanol	1%	24	7.2	200	2	2	1.00	2B
	1%	48	10.7	200	0	0	0.00	--
Positive Control								
Cyclophosphamide	60 µg	24	6.3	200	24	21	10.50	10B; 2IB; 4F; 5IF; 3E
Test Material								
Benzyl benzoate	30 µg	24	5.7	200	6	6	3.00	3B; 2IF; 1D
	100 µg	24	7.3	ND[d]	--	--	--	--
	250 µg	24	8.8	200	>9	5	2.50	1B; 3IF; 1MA
	500 µg	24	6.6	200	>7	3	1.50	1IF; 1MA; 1E
	250 µg	48	12.3	ND[d]	--	--	--	--
	500 µg	48	13.0	200	2	2	1.00	1B; 1IF

[a]Number of metaphases per 1000 cells scored per culture.

[b]Gaps excluded.

[c]Abbreviations used:

B = Break
F = Fragments
IF = Isofragment
IB = Isobreak
E = Exchange
MA = Multiple aberrations (cells with >5 aberrations; all observed aberrations were exchanges)

[d]ND = Not done.

There also appeared to be a slight increase in simple aberrations (i.e., breaks and fragments) at the 30- and 250-µg/mL treatment levels. In the absence of a significant effect on the percentage of cells with aberrations, the findings were not definitive evidence of clastogenesis; they were, however, unusual.

Based on the results, the study author concluded that benzyl benzoate was not clastogenic in this in vitro human lymphocyte cytogenetic assay.

D. REVIEWERS' DISCUSSION/CONCLUSIONS: We conclude that there was no evidence of a clastogenic response induced by nonactivated benzyl benzoate in human lymphocytes derived from a single donor. However, the biological significance of the rare complex aberrations at all S9-activated levels following the 24-hour harvest illustrates the rationale for conducting human lymphocyte cytogenetic assays with replicate cultures from different donors or performing independent experiments. We believe that the relevance, if any, of these results could have been clearly established either by the use of donor cells from a second source or the performance of a repeat test. We assess, therefore, that the data from the S9-activated phase of testing are inconclusive and that the study should be repeated.

E. QUALITY ASSURANCE MEASURES: Was test performed under GLPs? Yes. (A quality assurance statement was signed and dated June 18, 1991).

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 14-16.

APPENDIX A

MATERIALS AND METHODS
CBI pp. 14-16

benzyl benzoate

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